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Role of N-myc in the Developing Mouse Kidney

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N-myc is a transcription factor expressed in the developing metanephric kidney and other organs. In mice, complete disruption of the N-myc gene results in fetal death on the first day of renal organogenesis. In addition to the null N-myc allele, others have generated a hypomorphic N-myc allele. In this study, combinations of these mutant genes were used to demonstrate that reduction in N-myc protein levels correlate with fewer developing glomeruli and collecting ducts in embryonic kidney explants. Histological sections revealed that the mutant kidneys were hypoplastic with normal developing structures. The data indicate that the hypoplasia is due to a reduction in proliferation rather than an increase in apoptosis. Thus, N-myc loss causes a decrease in numbers of ureteric bud tips and developing glomeruli in explants and hypoplastic kidneys *in vivo*, in a dose-dependent manner.

Key Words: **N-myc; kidney; development; proliferation; apoptosis.**

INTRODUCTION

Bilateral renal hypoplasia is a leading cause of chronic renal failure in childhood, accounting for approximately 20% of all cases (Bernstein, 1992). At present, molecular mechanisms responsible for hypoplasia are unknown. Other congenital kidney abnormalities such as dysplasia or aplasia are reproduced in the mouse with targeted disruption of genes such as glial cell line-derived neurotrophic factor (Sanchez *et al.*, 1996) or c-ret (Schuchardt *et al.*, 1994). Mutations of other genes expressed in mouse embryonic kidneys do not display renal phenotypes (Davies and Bard, 1998). Disruption of N-myc leads to early embryonic lethality with uncertain effects on the kidney (Lipschutz, 1998).

Myc proteins are developmentally expressed basic helix-loop-helix leucine zipper transcription factors (reviewed in Henriksson and Lüscher, 1996; Grandori and Eisenman, 1997). c-myc was first identified as the chicken cellular homolog of oncogene v-myc (Henriksson and Lüscher, 1996). N-myc was later isolated from human neuroblastomas as an amplified gene having significant sequence homology with c-myc (Kohl *et al.*, 1983). Before binding to promoters, myc proteins form heterodimers with other factors and then either activate or repress transcription (Grandori and Eisenman, 1997). Although few myc target genes have been identified, the proteins appear to regulate cell growth, apoptosis, and transformation (Henriksson and Lüscher, 1996).

N-myc and c-myc differ in their expression patterns. Both appear in mouse embryonic tissues, but only c-myc is normally detected in the adult (Stanton and Parada, 1992). N-myc and c-myc are often coexpressed, but often with different levels of abundance. Mouse embryonic livers display high levels c-myc transcript, but little N-myc, while nervous tissues express abundant N-myc mRNA, but little or no c-myc (Stanton *et al.*, 1992). In other developing organs such as the lung and intestine, c-myc mRNA is restricted to mesenchymal cells and N-myc mRNA to epithelial cells (Stanton *et al.*, 1992). In the embryonic kidney, c-myc is detected in uninduced metanephric mesenchyme and N-myc in early induced mesenchymal condensates, while both are excluded from the ureteric bud epithelium (Patterson and Dressler, 1994).

Metanephric kidneys develop from two tissue precursors, the ureteric bud and the metanephric mesenchyme (reviewed in Davies and Bard, 1998). At gestational day 11.5 (E11.5) in the mouse, the ureteric bud appears as an evagination of the nephric duct in the region of the hindlimb and the metanephric mesenchyme as paired densities of mesen-

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chymal tissue adjacent to the ureteric bud (Davies and Bard, 1998). The two tissues then come into contact and begin a series of reciprocal inductive signals leading to the formation of the kidney (Davies and Bard, 1998). The mesenchyme stimulates the ureteric bud to begin branching successively (Davies and Bard, 1998). At each terminal tip, the ureteric bud induces local areas of mesenchyme to condense; these condensates eventually convert into the epithelium of the nephrons (Davies and Bard, 1998). The ureteric bud terminal tips then fuse with the nephrons and form the collecting ducts and ureters (Davies and Bard, 1998).

Researchers in the 1950s noted that explanted mouse ureteric bud and metanephric mesenchyme tissues will undergo apoptosis if grown separately (Grobstein, 1953). However, if dissected en bloc from the embryo after making contact, ureteric bud and mesenchymal tissues can grow and develop into a structure resembling a kidney (Grobstein, 1953). The ureteric bud will branch multiple times and the mesenchyme will be induced to form early epithelial nephron rudiments (Grobstein, 1953). These explants express histological markers, such as N-myc, in patterns consistent with embryonic kidneys *in vivo* (Saxen, 1987; Mugrauer and Ekblom, 1991).

Despite the high level of N-myc expression in the metanephric blastema, targeted disruption of N-myc leads to fetal demise at E11.5 in the mouse, so that the effects on the kidney could not be determined (Charron *et al.*, 1992; Stanton *et al.*, 1992). A hypomorphic N-myc mutation (H) has also been generated (Moens *et al.*, 1992). Mice homozygous for the hypomorphic mutation (HH) express approximately 25% of the N-myc protein levels found in wild-type littermates and die shortly after birth of lung hypoplasia (Moens *et al.*, 1992). When the null mutation is combined with the hypomorphic allele (NH), embryos die of apparent heart defects between E12.5 and E14.5 (Moens *et al.*, 1993). HH embryos were reported as having normal kidneys up until birth (Moens *et al.*, 1992); however, four NH embryos were described as having small kidneys prior to death at E14.5 (Moens *et al.*, 1993).

The purpose of our study was to examine the effects of N-myc mutations on the developing kidney. To circumvent problems with early embryonic death from the NN and NH mutations, we used embryonic kidney explants to characterize effects on the ureteric bud and metanephric mesenchyme. When possible, we also compared sizes and histology of N-myc mutant and control kidneys *in vivo* at later stages of development.

METHODS

Animals. Two- to three-month-old mice heterozygous for null (NW) and/or hypomorphic (HW) N-myc alleles were mated to generate embryos with NN, NH, or HH genotypes. The H allele was in an ICR mouse strain background. The N-myc null allele was in a combined C57BL/6 and 129Sv (approximately 50% each) mouse strain background, but was then backcrossed into the ICR strain three times. Subsequent NH embryos were approximately 93.75% ICR.

Explant studies. At E11.5, embryonic kidneys were dissected and cultured for 3 days in Dulbecco's modified Eagle medium (GIBCO) with 10% fetal bovine serum (GIBCO). Yolk sacs were saved for genotyping. Explants were then fixed in cold methanol (whole-mount studies) or frozen in OCT (Sakura Finetek, Inc.) embedding medium (cryosection studies).

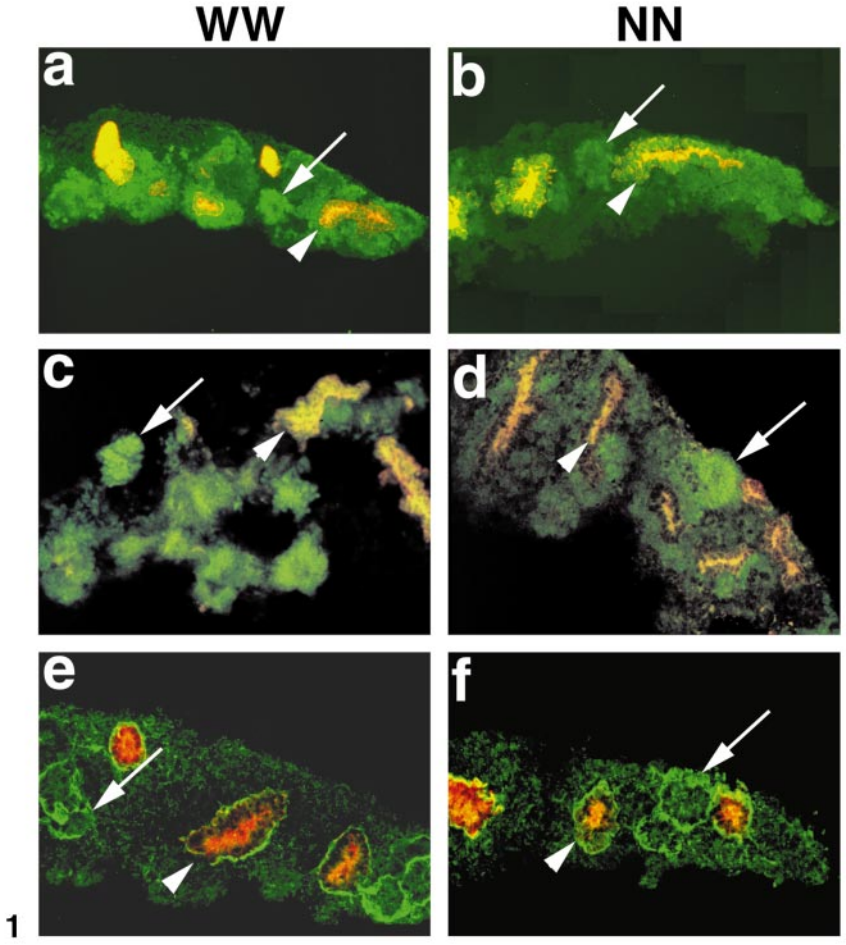
To genotype the explants, yolk sacs were digested overnight at 55°C with a solution containing 100 mM Tris–HCl, pH 8.5, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, and 100 μg/ml proteinase K (Sigma). DNA was precipitated and resuspended in 200 μl TE (25 mM Tris, pH 8.5, 1 mM EDTA). Samples were genotyped by polymerase chain reaction as previously reported (Moens *et al.*, 1993).

Whole-mount immunofluorescence was performed with mouse antibodies against pancytokeratins (Sigma) to label the ureteric bud or WT-1 (Santa Cruz) to label developing glomeruli (Cho *et al.*, 1998). Briefly, methanol-fixed explants were blocked in phosphate-buffered saline (PBS) with 2% milk and 0.5% Triton X for 1 h. Explants were labeled with mouse anti-pancytokeratins (1:8) and/or rabbit anti-WT-1 (1:100) in blocking solution. After being washed with blocking solution, the explants were incubated for 1 h in goat anti-mouse antibodies (for pancytokeratin) conjugated with Cy3 (1:500) (Jackson) or Cy2 (1:10) (Jackson) and/or goat anti-rabbit antibodies (for WT-1) conjugated with Cy3 (1:500) (Jackson). Representative samples were photographed with either a 35-mm or a digital camera (Optronics) mounted on a microscope (Leica or Olympus). The ureteric bud terminal tips and WT-1-labeled glomeruli were then counted with the operator blinded to the genotypes. Mean mutant bud tip and glomerular numbers were then compared with those of littermates by unpaired *t* tests ($P < 0.05$ was considered significant). Each experiment was repeated three times.

Explants were also cryosectioned at 7 μm and fixed in 4% paraformaldehyde in PBS. Samples were then dual labeled for 1 h with anti-pancytokeratins (1:8) to stain ureteric bud epithelium and anti-WT-1 (1:100), rabbit anti-laminin (1:100) (Sigma), or rabbit anti-pax2 (5 μg/ml) (Babco) to detect developing epithelial structures in the mesenchyme (Torres *et al.*, 1995; Cho *et al.*, 1998). After being washed in PBS, the slides were incubated for 30 min in a mixture of goat anti-mouse Cy3-conjugated antibodies (1:500) and goat anti-rabbit FITC- (1:25) or Cy2- (1:10) conjugated antibodies. Representative samples were photographed as above. Each experiment was repeated three times.

In vivo studies. E13.5 and E18.5 HH and viable E13.5 NH embryos or kidneys were obtained (along with yolk sacs for genotyping) and placed into Bouins solution (70% picric acid, 10% formalin, 5% acetic acid) overnight. Samples were washed daily with 70% ethanol for 4–6 days. Some of the E13.5 embryos were transverse-sectioned (5 μm—paraffin) and stained with hematoxylin and eosin (H&E) (as in Bates *et al.*, 1997). Some of the E18.5 HH and WW kidneys were longitudinally sectioned (5 μm) and H&E stained.

At E13.5, relative kidney lengths of NH and WW littermates ($N = 6$ each) and HH and WW littermates ($N = 8$ each) were compared from photographs. A ratio of NH or HH kidney length to WW littermate length was obtained. The means of the ratios of the NH–WW and the HH–WW lengths were calculated and compared (with WW–WW normalized to 1) by a one-way analysis of variance (ANOVA). E18.5, mean HH and WW kidney lengths ($N = 6$ each)



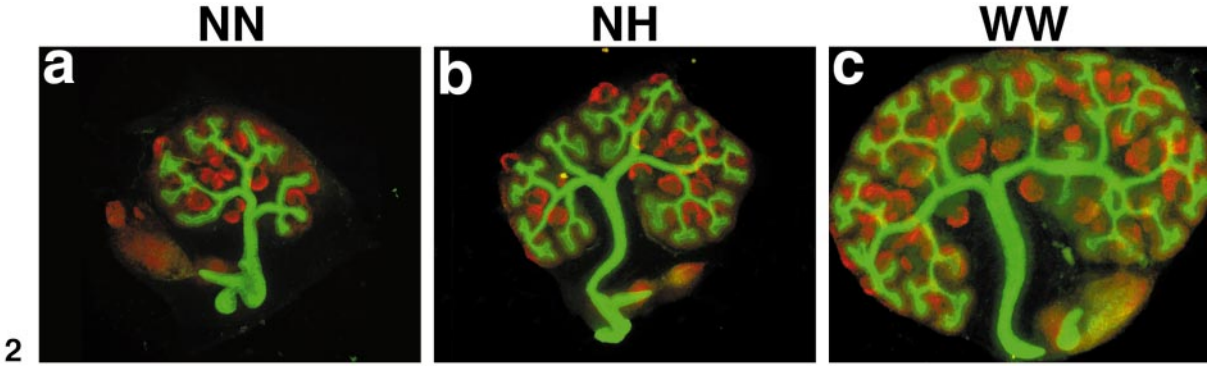


FIG. 1. Double-label immunofluorescence of wild-type (WW) kidney and N-myc null (NN) explant sections after 3 days in culture. (a and b) Pax-2 (green) and pancytokeratin (orange) antibody staining in wild-type (a) and null (b) explants. (c and d) WT-1 (green) and pancytokeratin (orange) antibody staining in wild-type (c) and null (d) explants. (e and f) Laminin (green) and pancytokeratin staining in wild-type (e) and null (f) explants (arrows, developing nephron epithelia derived from metanephric mesenchyme arrowheads, ureteric buds) (200× original magnifications).

FIG. 2. Representative whole-mount WT-1 (red) and pancytokeratin (green) immunofluorescence of N-myc mutant and wild-type kidney explants cultured for 3 days. (a) N-myc null explants. (b) N-myc null-hypomorph heterozygous explants. (c) Wild-type explants (100× original magnifications).

were compared by unpaired *t* tests ($P < 0.05$ was considered significant for *t* tests and ANOVA).

Mean whole embryo crown–rump lengths in E13.5 NH ($N = 8$) and WW ($N = 15$), E13.5 HH ($N = 8$) and WW ($N = 7$), and E18.5 HH and WW ($N = 5$ each) were compared by unpaired *t* tests ($P < 0.05$ was considered significant).

Cell proliferation/apoptosis assays. To measure apoptosis, E12.5 and E13.5 NH and WW embryo transverse sections (5 μm—paraffin) were assayed by terminal uridine triphosphate (UTP) end-labeling (TUNEL) (as in Farinas *et al.*, 1996). Briefly, sections were treated with proteinase K at 10 μg/ml for 10 and then 3% H_2O_2 for 5 min. After treatment with terminal transferase (TdT) buffer (Gibco) for 15 min, sections were incubated with 40 μM biotinylated UTP (Gibco) and 0.3 units/μl TdT (Gibco) for 1 h at 37°C (one slide without TdT added as negative control). Tissues were then washed with 2× SSC and then blocked with 2% horse serum in PBS. After washing, sections were incubated with an avidin:biotinylated horseradish peroxidase complex (ABC) (Vector Laboratories, Inc.) for 30 min at room temperature. The complex was visualized with DAB and the tissue lightly counterstained with hematoxylin. The experiments were performed three times.

To measure cell proliferation, bromodeoxyuridine (BrdU) assays were performed (as in Mokry and Nemecek, 1995) on E12.5 and E13.5 embryos whose mothers were intraperitoneally injected with 5 mg of BrdU 1 or 4 h prior to sacrifice. Transverse paraffin sections (5 μm) were incubated in 3% H_2O_2 for 30 min followed by pepsin at 200 μg/ml for 20 min. Samples were then bathed in 2 N HCl for 40 min followed by 0.1 M sodium borate, pH 8.5, for 10 min. Sections were blocked in 2% horse serum for 1 h and then incubated with mouse anti-BrdU antibodies (1:20) (Becton–Dickinson, Inc.) overnight at 4°C (one slide incubated without antibody as a negative control). After washes in PBS, samples were treated with goat anti-mouse antibody conjugated with biotin (1:400) (Vector Laboratories, Inc.) for 1 h. ABC solution was applied, followed by DAB and light hematoxylin staining.

To quantitate cell proliferation at E12.5, NH and WW littermates ($N = 6$ each) were serially cut and every fourth to fifth section (20–25 μm) was stained for BrdU as above (five sections total). Numbers of BrdU-positive (brown) and -negative (blue) cells were counted for both metanephric mesenchyme and epithelial ureteric bud. On average, a total of 4084 mesenchymal cells and 440 ureteric bud cells were counted for each WW embryo, while 3445 mesenchymal cells and 380 ureteric bud cells were counted for each NH embryo. Proliferation for each tissue type was calculated from the ratio of BrdU-positive to total nuclei (brown plus blue) in NH and WW. Mean percentages of proliferation were then compared with an unpaired *t* test ($P < 0.05$ was considered significant).

RESULTS

Explant Studies

Since N-myc is expressed in metanephric mesenchymal condensates, we hypothesized that interruption of the gene would inhibit the mesenchymal to epithelial conversion. N-myc null embryos die before this process occurs *in vivo;* therefore, we utilized embryonic kidney explants to test the hypothesis. Unfortunately, 60–70% of the null embryos harvested at E11.5 (first day of metanephric kidney formation) were undergoing reabsorption and we were unable to dissect out kidney anlage. The null explants we did obtain were smaller than their wild-type counterparts and did not increase in size significantly over the 3 days in culture, in contrast to the controls. After 3 days in culture, we fixed, cryosectioned, and immunostained the tissues with antibodies against pax-2, laminin, or WT-1 to identify developing nephron epithelia and pancytokeratins to visualize the ureteric bud (Fig. 1). Despite their smaller size, the null mutant explants labeled for pax-2, laminin, and WT-1, suggesting that the mesenchymal to epithelial conversion can occur in the absence of N-myc.

Since the viable N-myc null explants did not grow as well as their wild-type counterparts, we tested whether N-myc deletion affected ureteric bud branching morphogenesis and numbers of developing glomeruli. After 3 days in culture, we fixed explants and performed whole-mount immunofluorescence with antibodies against pancytokeratins and/or WT-1. N-myc null kidneys did demonstrate significant reductions in numbers of ureteric bud branches and developing glomeruli (Fig. 2a) relative to wild-type controls (Fig. 2c).

Since many of the 11.5-day null explants were smaller and possibly undergoing reabsorption at the time of dissection, we were concerned that the effects on ureteric bud branching and glomerular number were nonspecific. To circumvent this problem, we performed similar studies on explants dissected from embryos carrying one null (N) and one hypomorphic (H) allele. These compound heterozygotes possess approximately 15% N-myc protein levels relative to wild-type embryos (Moens *et al.*, 1993) and while sometimes smaller, they are otherwise indistinguishable from wild-type embryos at 11.5 days gestation (Moens *et al.*, 1993). Thus, at the time of dissection, we were unable to identify which of the embryos and explants were NH mutants. After 3 days in culture, we again performed whole-mount immunolabeling with antibodies against pancytokeratins and/or WT-1. The null-hypomorph (NH) mutant explants demonstrated many more ureteric bud branches and glomeruli than NN mutants, but fewer branches than their wild-type counterparts (Fig. 2). Although the NH and NN explants demonstrate progressively fewer ureteric bud branches than the wild-type, the overall pattern of branching appears intact (Fig. 2). Explants from other heterozygous embryos with one wild-type allele (NW and HW) were indistinguishable from wild type (not shown).

To quantitate the effects of the N-myc null-hypomorph mutation on the explants, we counted numbers of developing glomeruli and ureteric bud tips after WT-1 and pancytokeratin staining, respectively. The numbers of glomeruli and terminal tips did not vary among the wild type, the NW heterozygotes, or the HW heterozygotes (not shown) and therefore the controls included all three groups. The null-hypomorph mutants demonstrated a 20.3% reduction in mean numbers of developing glomeruli relative to controls [21.2 ± 0.7 versus 26.6 ± 0.5, respectively ($P < 0.0001$)] (Fig. 3b). Similarly, NH explants displayed 24.2% decrease

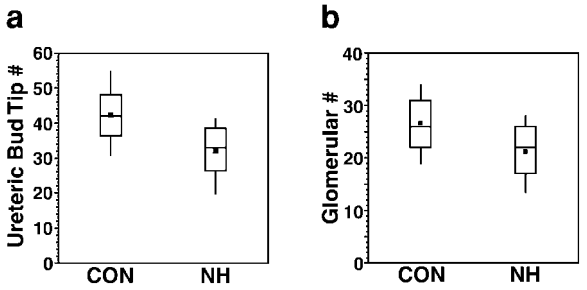


FIG. 3. Graphs comparing numbers of ureteric bud tips (a) and developing glomeruli (b) in N-myc null-hypomorphic (NH) and control (CON) explants after 3 days in culture. Mean numbers of CON and NH ureteric bud tips (control $N = 128$, mutant $N = 44$) and glomeruli (control $N = 167$, mutant $N = 60$) were significantly different (each with $P < 0.0001$). Squares represent the means, the ends of the vertical lines are outliers, and the three horizontal lines are the 25th, 50th, and 75th percentiles, respectively.

in mean ureteric bud tips relative to controls [32.0 ± 1.2 versus 42.3 ± 0.7, respectively ($P < 0.0001$)] (Fig. 3a). Interestingly, when the embryos were products of parents from different mouse strains (ICR crossed with a combined 129 Sv/C57BL6 strain) the reduction in NH mean ureteric bud tips increased to 39.9% relative to controls [21.8 ± 1.3 versus 36.3 ± 1.3, respectively ($P < 0.0001$)].

We also quantified the ureteric bud tip numbers in hypomorph-homozygous (HH) embryos. HH mutants were reported to be the same size as and indistinguishable from other littermates at E11.5 and to develop up until birth, when they die of respiratory failure (Moens *et al.*, 1992). Densitometry studies on Northern blots demonstrated that HH embryonic kidneys have 35% N-myc mRNA transcript levels relative to controls (Moens *et al.*, 1992). We dissected HH mutant embryonic kidneys at E11.5 and were unable to distinguish them from other littermates. After 3 days in culture, however, we found that the HH explants demonstrated a 18.4% reduction in ureteric bud tip numbers relative to controls [26.9 ± 1.1 versus 32.9 ± 0.8, respectively ($P < 0.0001$)].

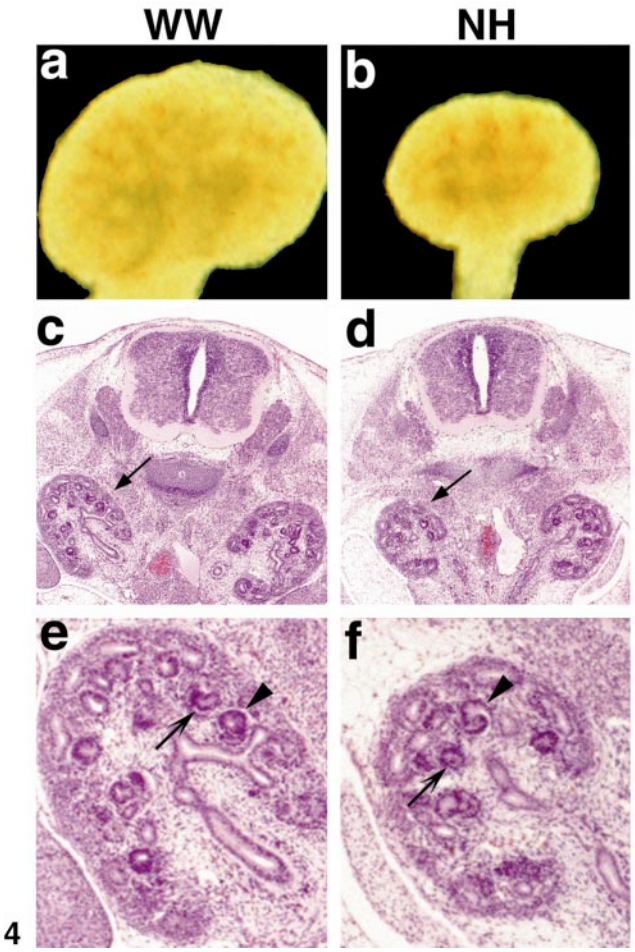
In Vivo Comparisons

Since the *in vitro* data suggested that N-myc expression levels correlated with ureteric bud branch and glomerular number, we tested whether reductions in N-myc affected kidney development *in vivo*. Although murine kidney development starts at E11.5, significant ureteric bud branching and early nephron formation does not begin until E13.5. Although NN embryos die at E11.5, most NH embryos are viable at E12.5 and a small number reach E14.5 (Moens *et al.*, 1993). We generated multiple litters from NW and HW matings and found that only 5–10% of the E13.5 NH mutants were viable. Similar to previous reports (Moens *et al.*, 1993), the viable NH mutants had cervical body wall edema in comparison to littermates. The mutants did appear slightly smaller than their littermates, but crown–rump lengths were not significantly different [9.50 ± 0.27 mm versus 9.98 ± 0.21 mm, respectively ($P = 0.19$)]. The NH embryos did not appear to be developmentally retarded.

We dissected whole kidneys and performed H&E stains on transverse sections of viable E13.5 NH mutants and their littermates. E13.5 null-hypomorph kidneys were significantly smaller than controls, with an average decrease in renal length of 24.5% ($P < 0.0001$) (Figs. 4a and 4b). Cross sections of the E13.5 embryos in the region of the kidneys confirmed that the volume of the renal parenchyma is reduced, but that normal developing structures are present (Figs. 4c–4f). Both mutant and control kidneys demonstrate developing S-shaped and comma bodies in the juxtamedullary cortex, while noncondensing mesenchyme predominates in the outer cortex. Branching ureteric bud structures also appear in both mutants and controls (Figs. 4c–4f).

We performed similar experiments on hypomorph-homozygous (HH) embryos, first at E13.5 and then again at E18.5. Previous reports indicated that from E12.5 until E18.5, the HH embryos were slightly smaller than littermates, but not developmentally retarded (Moens *et al.*, 1993). At E13.5, HH mutants were indistinguishable from viable E13.5 NH mutants, i.e., the E13.5 hypomorph homozygotes were slightly smaller than littermates, but crown–rump lengths were not significantly different [9.38 ± 0.16 mm versus 9.86 ± 0.21 mm, respectively ($P = 0.084$)] (not shown). HH mutant kidneys were smaller relative to littermate kidneys, although the average renal length was reduced by only 17.4% ($P < 0.0001$) (not shown). Furthermore, the decrease in kidney length relative to littermates was significantly greater in the null-hypomorph versus hypomorph homozygotes [27.5 ± 2.7% versus 17.4 ± 2.0%, respectively ($P < 0.05$)]. As with NH mutants, transverse sections of E13.5 HH embryos demonstrated a reduction in renal parenchymal volume, but normal developing structures (not shown).

At E18.5, HH mutants were still developmentally comparable to littermates, although the difference in embryo size was slightly larger than at E13.5 (Figs. 5a and 5b). E18.5 wild-type embryo crown–rump lengths measured on average 6.7% longer than HH crown–rump lengths [2.23 ± 0.034 cm versus 2.08 ± 0.015 cm, respectively ($P < 0.05$)]. Although the embryo length discrepancy was approximately 2% greater at E18.5 than at E13.5, the difference in kidney sizes was even more dramatic (Figs. 5c and 5d). E18.5 hypomorph-homozygous kidney lengths were on average 26.5% smaller than wild-type littermates (as opposed to 17.4% at E13.5) [2.00 ± 0.049 mm versus 2.72 ± 0.062 mm, respectively ($P < 0.0001$). Longitudinal sections of E18.5 HH kidneys revealed a normal architecture, but confirmed the vast reduction in size relative to littermates (Figs. 5e and 5f).



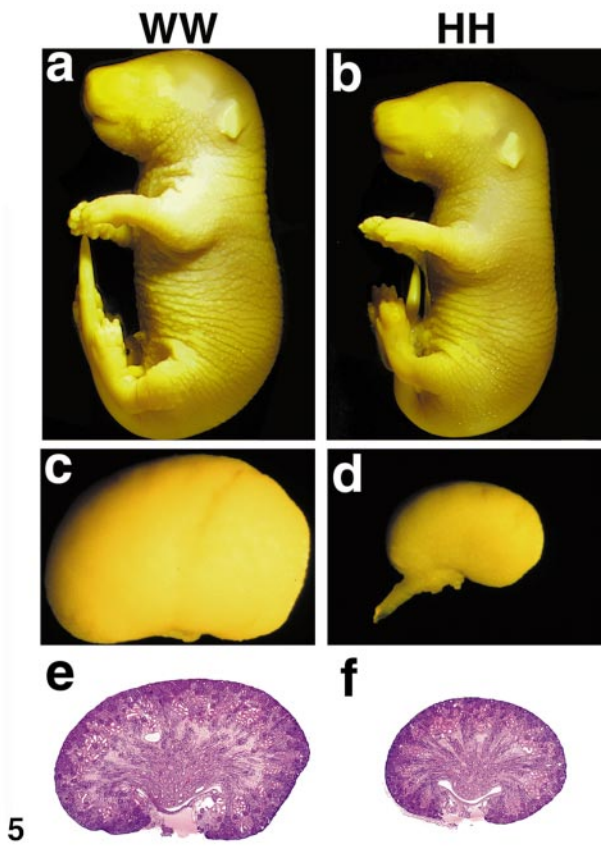


FIG. 4. Comparison of E13.5 N-myc null-hypomorph (NH) and wild-type (WW) kidneys. (a and b) Whole unstained kidneys dissected from wild-type (a) and null-hypomorph (b) embryos (100× original magnifications). (c–f) H&E stains of transverse sections through wild-type (c and e) and null-hypomorph (d and f) embryos in region of kidneys (arrows, kidneys; convex arrows, comma bodies; arrowheads, S-shaped bodies) (c and d, 40× original magnifications; e and f, 100× original magnifications)

FIG. 5. Comparison of E18.5 N-myc hypomorph homozygous (HH) and wild-type (WW) embryos and kidneys. (a and b) Wild-type (a) and hypomorph-homozygous (b) embryos (7× original magnifications). (c and d) Whole unstained kidneys dissected from wild-type (c) and hypomorph-homozygous (d) embryos (40× original magnifications). (e and f) H&E stains of longitudinal sections through wild-type (e) and hypomorph-homozygous (f) kidneys (40× original magnifications).

Proliferation and Apoptosis Studies

Since N-myc mutant kidneys appeared hypoplastic relative to controls, we tested whether N-myc reduction was associated with an increase in apoptosis and/or a decrease in proliferation in E12.5 and E13.5 NH embryos. For the apoptosis studies, we used the TUNEL assay. To test for proliferation, we determined percentage BrdU incorporation into embryos after injecting pregnant females with BrdU by intraperitoneal route 1 h prior to sacrifice.

The pattern of apoptosis in null-hypomorph and wild-type kidneys was similar, at both E12.5 (Figs. 6a and 6b) and E13.5 (not shown). The staining was sporadic and in the medullary and juxtamedullary regions of the kidneys (Figs. 6a and 6b) and did not appear in negative controls (not shown). In contrast, NH kidneys at both E12.5 and E13.5 demonstrated marked reductions in BrdU uptake throughout the developing cortex compared with wild-type littermates (Figs. 6c–6f). Similar data were obtained at E12.5 when BrdU was injected 4 h prior to sacrifice (not shown).

To quantitate the reduction in proliferation, we determined the percentage of BrdU-positive versus total cells in E12.5 NH mutant and wild-type littermates injected 1 h prior to harvest. We also tested whether the decreases in BrdU uptake were in the ureteric bud cells or in metanephric mesenchymal cells. Although embryonic kidneys are significantly larger at E12.5 than at E11.5, the metanephric

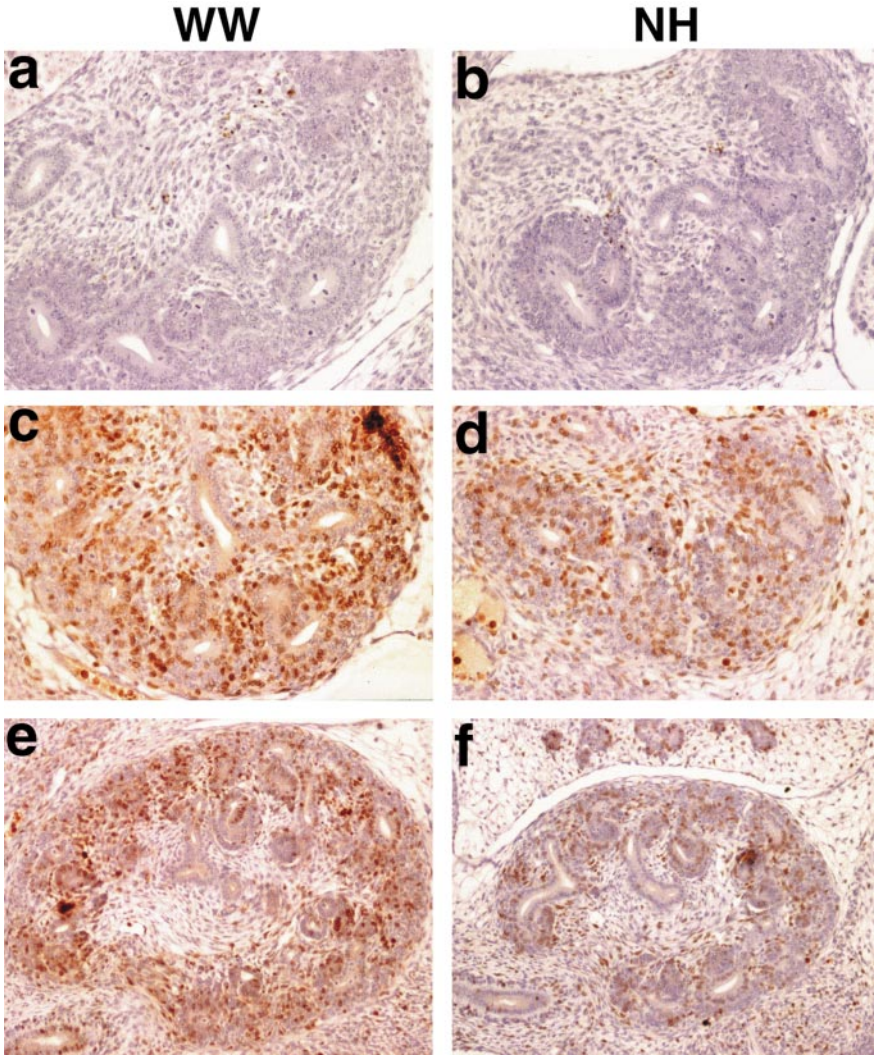


FIG. 6. Comparison of apoptosis and proliferation in N-myc null-hypomorph (NH) and wild-type (WW) embryonic transverse sections in region of kidneys. (a and b) TUNEL assays in E12.5 wild-type (a) and null-hypomorph (b) embryonic kidneys (100× original magnifications). (c–f) BrdU incorporation in E12.5 and E13.5 wild-type (c and e, respectively) and null-hypomorph (d and f, respectively) embryonic kidneys (c and d, 100× original magnifications; e and f, 40× original magnifications).

mesenchyme has not yet started forming epithelial structures of future nephrons (Davies and Bard, 1998); therefore, the epithelial ureteric bud cells are easy to distinguish from mesenchymal cells. Since only nuclei were stained, however, we were unable to distinguish between condensing and noncondensing metanephric mesenchymal tissues. We detected sharp reductions in both total metanephric mesenchymal and ureteric bud proliferation in E12.5 null-hypomorph mutants relative to controls (Fig. 7). Null-hypomorph kidney mesenchymal cells demonstrated a 25.3% decrease in BrdU staining in comparison to littermates [39.3 ± 1.29% versus 52.6 ± 0.75%, respectively ($P < 0.0001$)], while ureteric bud cell proliferation decreased by 21.8% [46.5 ± 2.86% versus 59.5 ± 1.59%, respectively ($P < 0.0001$)].

DISCUSSION

Our data provide strong evidence that N-myc has a pivotal role in kidney development. Although previous reports on N-myc mutations were unable to address the effects on the metanephric kidney due to early embryonic lethality (Stanton *et al.*, 1992a; Charron *et al.*, 1992), we used organ explants in part to circumvent this problem. Although complete disruption of N-myc does not prevent

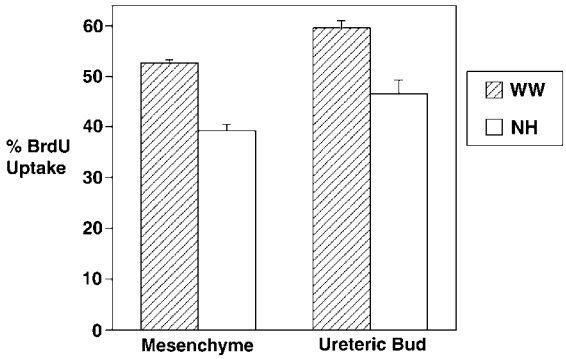


FIG. 7. Comparison of proliferation in metanephric mesenchyme and ureteric bud tissues of E12.5 null-hypomorph (NH) and wild-type (WW) embryonic kidneys. Significant differences were detected in both the metanephric mesenchymal cells ($P < 0.0001$) and the ureteric bud cells ($P < 0.005$).

the mesenchymal to epithelial conversion into early nephrons, the numbers of developing nephrons and ureteric bud tips were reduced dramatically. Using combinations of hypomorphic and null alleles, we demonstrated that progressive decreases in N-myc levels demonstrated more severe phenotypes, despite no apparent differences in explants at the time of dissection. *In vivo,* reduction in N-myc levels was associated with kidney hypoplasia disproportionate to the effects on the whole embryo. As with the explants, the expression level of N-myc correlated with the severity of the *in vivo* kidney phenotypes (although at E13.5, NH and HH embryos were of a similar size). Taken together, the explant and *in vivo* data support a direct role of N-myc in kidney development.

Explant Data

The mechanisms of the N-myc effects on the explants are unknown. The reduction in ureteric bud branching could be direct although a secondary mechanism is more likely given that N-myc expression has not been described in bud epithelium. The effect on glomerular number would more likely be direct given that N-myc is expressed in nephron precursor tissue. Alternatively, fewer glomeruli may be the result of the decrease in ureteric bud tip number. Regardless, these data are consistent with what has been reported in the mesonephric kidney, which develops earlier but in a manner analogous to the metanephric kidney. The original articles on N-myc deletion noted that mutant embryos had normal-appearing mesonephric nephrons, but a reduction in number (Stanton *et al.*, 1992a; Charron *et al.*, 1992).

The severity of the N-myc mutation in explants varies with the mouse strain. In a 50% ICR, 25% C57BL/6, and 25% 129Sv background, the null-hypomorph explants had an average of 39.9% fewer ureteric bud tips than controls after 3 days in culture. When the ICR background was increased to 93.75%, the decrease in NH ureteric bud branching was only 24.2%. This phenomenon has been reported for a variety of other targeted mutations (Ewart-Toland *et al.*, 1999; LeCouter *et al.*, 1998; Zhu *et al.*, 1998). For example, smad 3 null mutant mice develop colon adenocarcinoma 100% of the time in a pure 129Sv strain, but less than 30% of the time in a hybrid 129Sv/C57Bl/6 background (Zhu *et al.*, 1998). Thus, modifier genes in the 129Sv and/or C57BL/6 strain likely increase the severity of the N-myc mutations.

In Vivo Data

The effect of N-myc reduction on whole embryonic kidneys correlated well with the organ culture data. At E13.5, both the NH and the HH mutants had significant decreases in kidney size relative to littermates. Further, the kidney defect is hypoplasia (normal developing renal tissues with a reduction in mass), as opposed to dysplasia (abnormally forming kidney structures), as is often seen in other mutations such as with GDNF (Sanchez *et al.*, 1996) or c-ret (Schuchardt *et al.*, 1994). Hypoplasia would be the predicted *in vivo* phenotype given that N-myc mutant explants display normal-developing mesenchymal and ureteric bud structures, albeit with a reduction in numbers of those structures.

Proliferation/Apoptosis Data

The hypoplasia seen in N-myc mutant kidneys appears to be due to a decrease in cell proliferation and not to an increase in apoptosis. NH and wild-type E12.5 and E13.5 kidneys displayed similar patterns of apoptosis with sporadic labeling in the medullary and juxtamedullary regions (Fig. 6). The staining pattern is very similar to that of previously published reports examining apoptosis in developing kidney using TUNEL assays (Dudley and Robertson, 1997). Labeling in other developing organs, such as the dorsal root ganglion (not shown), was also similar to other published reports (Fariñas *et al.*, 1996). In contrast to the TUNEL staining, the differences in BrdU incorporation in NH mutant and control E12.5 and E13.5 kidneys are very apparent (Fig. 6). Furthermore, both the ureteric bud and the metanephric mesenchymal tissues in the E12.5 N-myc mutant kidneys demonstrated statistically significant decreases in proliferation (Fig. 7).

How N-myc mutations cause decreases in kidney tissue proliferation is unclear. One possibility is that a reduction in N-myc protein levels leads to downregulation of the cell cycle. Key mediators of the cell cycle such as ornithine decarboxylase and CDC25A contain putative myc binding elements in their promoters (Roussel, 1998). Other cell cycle components such as cyclins E and A, and CDC25B, are also thought to be regulated by members of the myc family (Roussel, 1998). N-myc may also directly or indirectly regulate growth factors important to kidney development, such as GDNF or its ligand c-ret. In either case, the effects of N-myc on kidney tissue proliferation are appar-

ently both autocrine and paracrine since both the ureteric bud and the metanephric mesenchyme are affected. How N-myc loss has non-cell-autonomous effects on ureteric bud development remains to be determined.

In summary, we have demonstrated that reductions in N-myc protein levels are associated with fewer ureteric bud branches and developing glomeruli in mouse embryonic kidney explants. *In vivo*, N-myc mutant embryos demonstrate hypoplastic kidneys. The effects of N-myc on the *in vitro* explants and the *in vivo* kidneys appear to be dose-dependant. Finally, the hypoplastic kidneys are the result of a decrease in cell proliferation and not to an increase in apoptosis.

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